Here's to the losers: evolvable residents accelerate the evolution of high-fitness invaders

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Abstract: Recent work has shown that evolvability plays a key role in determining the long-term population dynamics of asexual clones. However, simple considerations suggest that the evolvability of a focal lineage of bacteria should also be influenced by the evolvability of its competitors. First, evolvable competitors should accelerate evolution by impeding the fixation of the focal lineage through a clonal interference–like mechanism. Second, evolvable competitors should increase the strength of selection by rapidly degrading the environment, increasing selection for adaptive mutations. Here we tested these ideas by allowing a high-fitness clone of the bacterium Pseudomonas aeruginosa to invade populations of two low-fitness resident clones that differ in their evolvability. Both competition from mutations in the resident lineage and environmental degradation lead to faster adaptation in the invader through fixing single mutations with a greater fitness advantage. The results suggest that competition from mutations in both the successful invader and the unsuccessful resident shapes the adaptive trajectory of the invader through both direct competition and indirect environmental effects. Therefore, to predict evolutionary outcomes, it will be necessary to consider the evolvability of all members of the community and the effects of adaptation on the quality of the environment. This is particularly relevant to mixed microbial communities where lineages differ in their adaptive potential, a common feature of chronic infections.

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Abstract

Recent work has shown that evolvability plays a key role in determining the long-term population dynamics of asexual clones. However, simple considerations suggest that the evolvability of a focal lineage of bacteria should also be influenced by the evolvability of its competitors. First, evolvable competitors should accelerate evolution by impeding the fixation of the focal lineage

through a clonal interference-like mechanism. Second, evolvable competitors should increase the strength of selection by rapidly degrading the environment, increasing selection for adaptive mutations. Here we tested these ideas by allowing a high fitness clone of the bacterium *Pseudomonas aeruginosa* to invade into populations of two low fitness resident clones that differ in their evolvability. Both competition from mutations in the resident lineage and environmental degradation lead to faster adaptation in the invader through fixing single mutations with a greater fitness advantage. The results suggest that competition from mutations in both the successful invader and unsuccessful resident shapes the adaptive trajectory of the invader, both through direct competition and indirect environmental effects. Therefore, to predict evolutionary outcomes, it will be necessary to consider the evolvability of all members of the community and the effects of adaptation on the quality of the environment. This is particularly relevant to mixed microbial communities where lineages differ in their adaptive potential, a common feature of chronic infections.

Keywords: experimental evolution, epistasis, whole genome sequencing, *Pseudomonas aeruginosa*

1 Introduction

In classical population genetics, evolutionary history is always written by the winners. When observing a population in its current state, we can only recover information about the lineages that survived natural selection. Consequently, little attention has been given to the role of the 'losers' of adaptation, lineages

that ultimately go extinct. Recent work has called into question the generality of the strong selection-weak mutation (SWMM) model of adaptation, where selective sweeps of beneficial mutations are brief and infrequent (Sniegowski and Gerrish, 2010). Next-generation sequencing of entire adapting populations has demonstrated that multiple beneficial mutations compete for fixation within a common population (Lang et al., 2013; Lieberman et al., 2014), which leads to an increase in the average fitness of the ultimate winner (De Visser and Rozen, 2006).

Under these conditions, a key determinant of the evolutionary fate of competing lineages is their 'evolvability'. Evolvability is defined as the extent to which a population can adapt to a fitness challenge, such as a novel environment, a deleterious mutation, or other stressor (Díaz Arenas and Cooper, 2013), over a given amount of time. Evolvability can be measured by observing the ability of a population to increase in fitness over a fixed period of time. Recent results have shown that a strain with lower initial fitness but greater evolvability can become the eventual 'winner' of an evolutionary contest between competing strains (Woods et al., 2011), but little is known about how the evolvability of the loser lineages influences the fitness of the winner.

Population invasions are a classic example from evolutionary ecology where the 'winners' and 'losers' are clearly defined. In this scenario, an initially low-frequency and high-fitness invader displaces a high-frequency and low-fitness resident population. Previous work has shown that interactions between invader and resident may spur evolution, and that resident evolvability can determine its resistance to invasion (Sakai et al., 2001; Gifford and MacLean, 2013). Given that an invader does not immediately replace the resident, com-

petition between adapting resident and invader can act like a 'selective sieve' on the fitness of the invader lineage, where high-evolvability residents produce mutations that can outcompete weakly beneficial mutations in the invader. Although the resident is ultimately lost from the population, its evolvability may nevertheless indirectly influence invader mean fitness by eliminating relatively low fitness invader lineages in a mechanism analogous to clonal interference (De Visser and Rozen, 2006). This argument predicts that invaders in high-evolvability resident populations should have higher mean fitness if they successfully fix, but have an overall lower success rate, compared to invaders in low-evolvability resident populations.

Separate from the direct competitive effects, resident evolvability could also influence invader adaptation through increased environmental degradation associated with adaptation to exploiting a common environment. As the resident increases its ability to consume the available resources, the total pool of resources is reduced, therefore increasing selection pressure on the invading lineage. This indirectly alters the selective advantage associated with beneficial mutations in the invader lineage, and consequently changes their rate of fixation. A resident that has higher evolvability is expected to deplete resources faster as it adapts faster, and thus should put greater pressure on its invader to adapt. Consequently, mutations in the invader would be expected to sweep to fixation faster when in the presence of a resident that degrades the environment more, if those mutations provide a greater relative selective advantage in depleted environments. This scenario is a manifestation of the Red Queen effect, which is known to increase the rate of evolution (Maynard Smith, 1976*a*,*b*).

Using strains of the bacterium *Pseudomonas aeruginosa*, we tested the effect of resident evolvability on invader adaptation by experimentally invading resident populations with a high-fitness invader. The resident populations consisted of two rifampicin-resistant genotypes previously shown to have equally-large fitness costs but differ in their resistance to invasion and their evolvability, as measured by a 30% difference in final fitness following 300 generations of selection (Gifford and MacLean, 2013). The invading lineage was a rifampicin-sensitive PA01 strain with a large fitness advantage over the resistant strains. Due to the high cost of rifampicin resistance, the invader ought to have rapidly swept to fixation without the need for additional mutations, but this did not occur due to adaptation by the residents. Previous work has shown that the resident with higher evolvability is less susceptible to invasion (Gifford and MacLean, 2013), but paradoxically, the invader tended to fix faster in highly-evolvable resident populations (Figure 1). Our data suggest that competition from and environmental degradation caused by the adapting resident both shaped adaptation of the invader by altering fixation probabilities, mean fitness and time to fixation. Our results suggest that resident evolvability is an important factor in determining invasion success, independently of the initial fitness differential between resident and invader.

2 Materials and methods

2.1 Strains and growth conditions

The 'resident' populations were comprised of one of two rifampicin-resistant mutants derived from a *P. aeruginosa* PA01 strain from MacLean and Buckling (2009): S536F (high-evolvability resident, 'RH') and +P518 (low-evolvability resident, 'RL'). The 'invader' strain was a PA01 strain marked with green fluorescent protein production and gentamicin resistance (gfp-PA01). A PA01 strain with the *luxCDABE* operon for luminescence production and gentamicin resistance was used for growth rate assays (lux-PA01). Both gfp-PA01 and lux-PA01 were made using the mini-Tn7 insertion protocol (Choi and Schweizer, 2006).

Strains were grown in liquid M9-serine at 37 °C as the selection environment [3.75 g/l (3.57×10^{-2} mol/l) L-serine, 1 g/l NH_4Cl, 6 g/l Na_2HPO_4, 3 g/l, KH_2PO_4, 0.5 g/l NaCl, and 1 ml of 1 mol/l $MgSO_4$ added post autoclaving; this corresponds to 35.7 mM L-serine with final pH approximately 7.4]. This environment was chosen because previous work showed that the RH and RL strains had initially equivalent fitness costs of rifampicin-resistance, but differed in evolvability as defined by their ability to adapt (Gifford and MacLean, 2013). Using a defined medium also permitted experimental manipulation of the carbon resource availability (see section 'Environmental degradation').

2.2 Resident population invasion

The population invasion experiment was conducted as part of an earlier work (Gifford and MacLean, 2013). gfp-PA01 was invaded at low initial frequency into dense RH and RL populations. An average of 0.37 invader cells were introduced into 384 RH and 384 RL populations, each consisting of 5.25×10^4 resident cells. A 1 µl sample of each population was transferred to 200 µl fresh medium every 24 h for 40 days. The transfer protocol corresponds to a minimum of six to seven doublings per transfer. Presence of the invader was tracked by selective plating on gentamicin. An invader was 'detectable' if it surpassed a frequency of 0.0005%, and 'successful' if surpassed a frequency of 99.975%, at which point it was stored at −80°C. This resulted in 38 RH and 54 RL populations where an invader reached detectable frequency. From those reaching detectable frequency, an invader successfully fixed in 25 RH and 45 RL populations. From these successful invaders, 15 from distinct RH populations and 15 from distinct RL populations were randomly chosen for competitive fitness and genotype analysis.

2.3 Competitive fitness assays

Successful invaders and the PA01 wild-type were first streaked out from freezer stocks on LB agar (LB agar-Miller: Fisher Scientific, UK) to single colonies. Cells were inoculated into 200 µl of M9-serine and were allowed two 24 h growth periods to physiologically recover from storage. Following the second growth period, 50 µl of each evolved invader culture was mixed with either 50 µl wild-type PA01 culture or ancestral resident culture. The mixtures were

diluted 1 µl into 200 µl M9-serine and grown for 24 hours at 37 °C.

Marker frequencies were measured using a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA). Mixed cultures were not diluted prior to measurement. Cells were distinguished from noise and debris by a gate of forward-scatter-height (FSC-H) $> 10,000$ and side-scatter-height (SSC-H) > 8000 (values are dimensionless and device-specific). Cell marker type (gfp or unlabelled) was determined by fluorescence intensity when excited by a 488 nm laser and passed through a 530 ± 30 nm filter. We measured 10 µl of sample from each competition. Fitness was calculated as $\log_2(F_f/F_i)/\log_2(U_f/U_i)$, where F and U denote fluorescent and unlabelled proportions and subscript i and f denote initial and final proportions. Competitive fitness data are deposited in Dryad (http://dx.doi.org/10.5061/dryad.v051c).

2.4 Environmental degradation

To determine whether environmental degradation contributed to selection for beneficial mutations, we first determined the effect of a degraded environment on the lux-PA01 tester strain. We simulated environmental degradation by varying the concentration of L-serine and measuring the maximum growth rate of the lux-PA01 tester strain ($v_{\max}$) in the presence of the ancestral resident (20% tester to 80% resident). We chose luminescence as a marker for measuring the growth rate of a single strain in a mixed population because there is little signal interference from the resident. L-serine concentration was set at 10%, 25%, 50%, and 75% of the selection environment in 200 µl medium (corresponding to 3.57–27 mM L-serine) To enhance signal detection, we used

a white opaque 96-well microtitre plate. Total luminescence (captured in 3 second intervals for each well) was measured every 5 minutes for the first 8 h, then every 30 minutes for a further 16 h using the FLUOstar OPTIMA (BMG Labtech, Offenburg, Germany). Maximum growth rate was estimated by fitting Gompertz growth curves to log-transformed luminescence using the 'grofit' package (Kahm et al., 2010) in R 3.0.1 (R Core Team, 2013). Gompertz growth curves provided a better fit than logistic curves, but fitting either returned similar parameter estimates, as previously demonstrated by Shi and Xia (2003). We transformed L-serine concentration into a measure of environmental degradation, $E = [1/(\%\text{ L-serine}) - 1]/10$ to linearize the relationship with growth rate (the constant divisor is to put E on a convenient scale). Environmental degradation ranges from 0 (a pristine environment) to infinity (completely devoid of resources).

To determine the effect of environmental degradation on competitive fitness, we measured fitness of the evolved invaders in a pristine ($E = 0$) and degraded ($E = 0.9$) environment, with the competitive fitness assay method using flow cytometry described in the previous section. Environmental degradation data are deposited in Dryad (http://dx.doi.org/10.5061/dryad.v051c).

2.5 DNA extraction and quality assessment

We extracted genomic DNA (gDNA) from individual colonies of successful invaders. DNA extractions were performed with the Promega Wizard Genomic DNA Purification Kit (Promega, UK). gDNA concentration and quality was assessed with spectrophotometry (Nanodrop, Thermo Fisher Scientific, UK) and

the QuantiFluor dsDNA System (Promega, UK) prior to sequencing. Full details can be found in the online supplementary materials.

2.6 Sequencing and analysis

The whole genome sequencing pipeline is essentially as described in San Millan et al. (2014). Whole genome sequencing was performed by the Wellcome Trust Centre for Human Genetics (Oxford, UK) using the Illumina hiseq 2000 platform with 100 bp paired-end reads. FASTQ files are deposited in NCBI Sequence Read Archive (SRP053291). Initial filtering of the reads was done using NIH QC Toolkit (Patel and Jain, 2012). 5' or 3' ends were trimmed if the Phred quality score was less than 20. Reads were discarded if they were $<$ 50 bp after trimming, if $>$ 2% of bases were ambiguous, or if more than 20% of bases had a Phred score $<$ 20. The filtered reads were mapped to the *P. aeruginosa* PA01 reference genome (NC_002516.2) using BWA. We processed the mapped reads to increase the quality of the variant calling: 1) reads with multiple best hits were discarded; 2) duplicated reads were discarded using MarkDuplicates from the Picard package (http://picard.sourceforge.net); 3) reads around indels were locally realigned using RealignerTargetCreator and IndelRealigner from the GATK package to correct for misalignment; and 4) mate pairs were sorted using FixMateInformation in the Picard package. Variant calling was performed using two tools: GATK's Unified Genotyper (DePristo et al., 2011) and Samtools's mpileup (Li et al., 2009). VCFtools (vcfannotate, Danecek et al., 2011) and GATK toolkit (VariantFiltration, DePristo et al., 2011) were used to filter the raw variants for strand bias, end distance

bias, base quality bias, SNPs around gaps, low coverage and erroneously high coverage. High quality variants not filtered were annotated using SnpEff (Cingolani et al., 2012).

Structural variants were detected using three approaches. First, BreakDancer (Chen et al., 2009) was used to predict deletions, insertions, inversions, and translocations using deviations in the separation or orientation of mapped read pairs. Pindel (Ye et al., 2009) was used to infer deletions, short insertions, long insertions, inversions, tandem duplications, and breakpoints using a split-read approach (the output of BreakDancer was also fed to Pindel to improve its output). Finally, Control-FREEC (Boeva et al., 2011) was used to detect copy number variants (CNVs). Control-FREEC finds CNVs using depth-of-coverage (normalized by GC-content). Regions of low mappability were excluded by supplying Control-FREEC with mappability tracks generated by gem-mappability (GEM library, Marco-Sola et al., 2012). Two published pipelines, CORTEX (Iqbal et al., 2012) and BRESEQ (Barrick et al., 2009), were additionally used to detect SNPs and structural variants but no new variants were found. Finally, de novo assembly of the unmapped reads to find novel sequences not found in the reference genome was also performed using Velvet (Zerbino and Birney, 2008).

3 Results

3.1 Resident and invader fitness

Residents initially had approximately equal fitness (0.820 ± 0.053 vs. 0.784 ± 0.031, t-test: $t_{18} = 0.58$, $p = 0.7$), but differed in evolvability (measured by fitness after 300 generations 1.19 ± 0.037 vs. 1.02 ± 0.059, t-test: $t_{18} = 2.44$, $p = 0.025$). Previous work has shown that high evolvability residents (RH) are more resistant to invasion (Gifford and MacLean, 2013), but that invaders in those populations fixed faster than in low evolvability residents (RL, Figure 1). To determine if this resulted from adaptation of the invader strain, we measured the adaptation of invaders following a successful invasion into RH and RL populations. We found that mean fitness of invaders was higher following invasion into the RH populations (1.03 ± 0.03 SE v.s. 1.12 ± 0.03 SE; two-sample t-test: $t_{36} = -2.3$, $p = 0.03$). To determine the underlying molecular basis of this difference, we performed whole genome sequencing on a total of 30 successful invaders, split between those that had invaded 15 distinct RH populations and 15 distinct RL populations. Sequencing revealed that invaders in both populations fixed similar numbers of mutations. The number of successful invaders that had either acquired one or more mutations (n=8), or remained wild-type (n=7), was identical in RH and RL populations (Fisher's exact test, odds ratio=1, $p > 0.99$). The average number of mutations fixed did not differ between RH and RL (Poisson GLM: RH vs. RL $z = 0.224$, $p = 0.8$, Table 1, Table S1). SNPs in gene PA2449 (encoding a transcriptional regulator) were the most common in invaders from both resident populations, with five mutations each.

Invaders lacking a mutation did not differ significantly in fitness from the ancestral invader, suggesting that few, if any, important beneficial mutations were missed by the sequencing pipeline (ANOVA: $F_{2,21} = 1.57$, $p = 0.23$). Mutations were associated with 5% average benefit in RL invaders and 19% average benefit in RH invaders (Figure 2). Qualitatively similar results were obtained when competing against the ancestral residents (Figure S1).Over the ancestral invader's fitness, the increase was significant for RH invaders (1.19 ± 0.041 SE; two-sample t-test: $t_{16} = -4.2$, $p < 0.001$), but non-significant for RL invaders (1.05 ± 0.049 SE; two-sample t-test: $t_{16} = -0.9$, $p = 0.37$). Mutant invaders in RH were also more fit on average than mutant invaders in RL (two-sample t-test: $t_{14} = -2.24$, $p = 0.042$).

Examining the adaptive potential of the residents sheds light on the likely mechanism responsible for the higher fitness gains made by invaders in the RH populations. Mean fitness of adapted invaders closely matched the fitness gains achievable by the resident they invaded (Figure 2: fitness of successful invader relative to fitness of evolved resident: invader vs RL mean 1.04 ± 0.049 vs. 1.02 ± 0.059: $t_1 4 = 0.26$, $p = 0.8$; invader vs RH mean $= 1.19 \pm 0.041$ vs. $= 1.19 \pm 0.037$: $t_1 4 = 0.02$, $p = 0.99$). This suggests that competitive interference from mutations in the evolving resident was a barrier to fixation of the ancestral invader, and that weakly beneficial mutations were prevented from fixing in the invader in RH populations.

3.2 Environmental degradation

For environmental degradation to affect invader adaptation favourably, two conditions must be met: the presence of an adapted resident must degrade the environment, and mutant invaders must have a larger fitness benefit when the environment is degraded. To evaluate these conditions, we measured the effect of environmental degradation on invader growth rate, the extent of environmental degradation caused by the presence of an evolved resident, and the fitness benefit conferred by mutations in the invader lineage in an artificially-degraded environment. In an experiment where we manipulated environmental degradation by changing nutrient availability, growth rate of a tester strain (lux-PA01, isogenic to the invader except at the mini-Tn7 insertion site) was significantly reduced in poor-quality environments (Figure 3A, Type II ANOVA: environmental degradation: $F_{1,7} = 7.58$, $p = 0.03$; resident genotype: $F_{1,7} = 14.5$, $p = 0.01$). Similarly, the presence of an evolved resident reduced the growth rate of the tester strain, thus we used tester strain growth rate as an indicator of 'effective environmental degradation' caused by an evolved resident. The reduction in growth rate correlated with the fitness of the evolved resident (Figure 3B, linear regression: intercept $= 0.09$, slope $= 1.35$, $R^2 = 0.35$, $F_{1,24} = 18.3$, $p = 0.0001$). Hence, invaders in evolving RH populations likely experienced a worse environment than those in evolving RL populations.

To evaluate the second condition, that mutant invaders have a greater benefit when the environment is degraded, we measured the benefit of the mutations acquired by the invader lineage in a poor quality environment. If muta-

tions confer a larger benefit over the ancestral invader in poor quality environments, invader mutations in the RH populations should fix faster than in RL populations. Competitive fitness of the beneficial mutations in the invader lineage was elevated in the degraded environment for mutant invaders from both resident populations (Figure 3C, Type II ANOVA: environmental degradation: $F_{1,75} = 301$, $p < 0.0001$; resident genotype: $F_{1,75} = 16.9$, $p < 0.0001$). However, the greater extent of environmental degradation caused by the evolved RH resident in the previous assay suggests that beneficial mutations should sweep faster in RH populations. Consistent with this finding, average fixation time of mutations in the invader was significantly lower in RH populations (Figure 1, 99 vs 153 generations, GLM: intercept $= 3.14 \pm 0.07$, RH $= -0.43 \pm 0.12$, $p < 0.001$). The reduced fixation time for invaders in RH was not driven by their increased fitness, as fitness and fixation time were not significantly related (linear regression: fixation time $t_{13} = -0.09$, $p = 0.9$).

4 Discussion

In this study, we investigated what effect the evolvability of a resident population has on the adaptation of a lineage invading into that population. Our data suggests that residents with higher evolvability can select for increased adaptation in the invader, even if the invader has initially higher fitness than the resident. Adapting residents influenced the adaptation of the invader both through competition from beneficial mutations and environmental degradation associated with residents adapting to exploit the environment.

Competition from beneficial mutations newly arising in the resident lin-

eages is a likely driver of invader adaptation; this is indicated by evolved invader fitness closely matching the evolvability of the resident (Figure 2), indicating that invaders needed to surpass the adapting residents' fitness through acquiring beneficial mutations. Note that this competitive effect does not change the characteristics of mutations available to the invader. Instead, it increases mean fitness of invaders that are ultimately successful by eliminating invader lineages with relatively low fitness. The requirement to wait for large-effect beneficial mutations to arise is consistent with the observed lower probability of success of invaders in RH populations (previously described in Gifford and MacLean, 2013, Figure 1B). Although theory (e.g. Rozen et al., 2002; Park and Krug, 2007) and data (e.g. Miralles et al., 1999; De Visser and Rozen, 2006) have previously demonstrated that competition between mutations in clonal lineages can lead to accelerated adaptation, ours is the first evidence that competition from mutations in competitors that are initially lower in fitness also influences the fitness effect of mutations that fix. The results imply that the evolutionary history of populations is not written only by the winners, but by the losers as well. In predicting how resident evolvability would affect the fitness of invaders, we have neglected any clonal interference effect from the presence of multiple beneficial mutations in different invader lineages. This is a reasonable assumption because the invader was introduced at low frequency (1 in 5.25×10^4, or probabilistically less than four cells, Gifford and MacLean, 2013) and at any rate, the effect should be independent of resident genotype. However, although competitive interference from the RH resident was higher than from the RL resident, the strength of interference would likely be even greater in a pure population of the invader, as the average fitness of mutants

would be greater. Consequently, mean fitness of the evolving invader could potentially reach higher fitness in the same span of time had it been adapting to the environment on its own.

Resource depletion was also investigated as a mechanism through which resident evolvability can influence invader adaptation. Environmental stress associated with environmental change is a driver of adaptation for invaders in natural populations (Gilchrist and Lee, 2007). Our results suggest that stress generated by the residents depleting the pool of resources can increase the strength of selection on the invader (Figure 2). The presence of an evolved resident decreased the apparent environmental quality experienced by the invader, and evolved invader fitness was higher in experimentally-degraded environments relative to pristine ones (Figure 3B and C). This suggests that decreases in resource availability induced by the adapting resident did not impose a new selection pressure on the invader, but merely made selection for L-serine consumption stronger. Consistent with these results, we found that invader mutations fixed faster in the RH populations (Figure 1), even though there was no correlation between invader fitness and fixation time. Here, we have assumed that resource availability is the greatest contributor of environmental quality. In general, the accumulation of waste products and active toxification are thought to be important determinants of environmental quality, but were unlikely to factor in to this experiment. Although ammonia, NH_3, is a waste product of L-serine catabolism (Hofmeister and Buckler, 1993), the concentrations produced, even if all L-serine was consumed, would be buffered as lower-toxicity ammonium ion, NH_4^+ (only 1×10^3 mol/l would remain as ammonia given the pH and temperature conditions, Emerson et al., 1975). Al-

though *P. aeruginosa* produces a wide variety of anti-competitor compounds, they are unlikely to be effective as invader and resident share a common genetic background (Inglis et al., 2011; Bucci et al., 2011; Schoustra et al., 2012). Likewise, cross-feeding is another important interaction between microbes that has been observed to evolve in vitro (Rozen and Lenski, 2000). However, given the short time scale of our experiment, the carbon source used (L-serine is converted to pyruvate in a single reaction step) and the nature of the mutations observed in the invader, it is unlikely to contribute here.

The sequencing data indicate that the difference in invader fitness manifested as a tendency to fix larger beneficial mutations, rather than more, as invaders in RH populations did not fix more mutations (Table 1). This is consistent with invaders in both populations drawing from the same distribution of beneficial mutations with the same mutation rate, but in RH populations a larger portion of the left-hand of the distribution is metaphorically 'covered up' by competitive interference, permitting fixation only of large-effect mutations and not small-effect mutations in the invader. Of the genes with mutations, only one is presently known to be important for L-serine metabolism. Ten mutations were found in gene PA2449, which is thought to be an enhancer binding protein controlling transcription of genes in the L-glycine and L-serine metabolism pathway (Lundgren et al., 2013). As PA2449 knockouts have impaired ability to metabolize L-serine, these mutations are not likely to cause loss of gene function.

These results suggest that to predict adaptive evolution, the evolvability of all members of the community must be considered, not only the lineage that becomes the 'eventual winner'. Evolutionary history, in terms of the final fit-

ness of the lineage that ultimately fixed, can be influenced by the competitors that are lost to selection. This suggests that adaptive outcomes will depend not only on competition from initially competing lineages, but also from their descendants, in a mechanism analogous to clonal interference. Additionally, it may be necessary to incorporate the effects of environmental change as lineages adapt to and degrade the environment, as environmental degradation can influence the fitness advantages of lineages in the population. This will require further studies of adaptive evolution in both the winners and losers of adaptation (such as Woods et al., 2011), as well as detailed studies on how adaptation changes environmental quality through time. These findings are especially relevant to predicting the evolutionary course of chronic bacterial infections, such as in the lungs of individuals with cystic fibrosis, where mixed communities are likely and mutators with increased evolvability are commonly found (Oliver et al., 2000; Harrison, 2007; Lieberman et al., 2014).

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6 Authors' contributions

Conceived the experiment: DRG and RCM. Conducted the lab work: DRG and MK. Analyzed whole genome sequence data: MT-R. Analyzed data and wrote the manuscript: DRG and RCM. All authors commented on and approved the final manuscript.

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7 Online supplemental methods

7.1 DNA extraction and quality assessment

DNA extractions were performed with the Promega Wizard Genomic DNA Purification Kit (Promega, UK). First, single colonies were isolated from LB agar plates and grown overnight in liquid LB. 1 ml of dense culture was centrifuged at maximum speed (13,000 rpm) for 2 min on a microcentrifuge. The supernatant was removed and the pelleted cells mixed with 600 µl nuclei lysis solution. Cells were then incubated for 5 min at 80°C and then cooled to room temperature. RNA in the cell lysate was degraded by adding 3 µl of RNase solution, mixing by inverting and incubation for 15-60 min at 37°C. Proteins were precipitated by adding 200 µl protein precipitate solution to the cell lysate and vortexing at maximum speed for a minimum of 30 s, followed by incubation on ice for 10 min and centrifuging at maximum speed for 10 min. 400 µl of supernatant containing DNA was recovered by pipetting and mixed with 600 µl isopropanol, followed by centrifuging at maximum speed to pellet the gDNA. The isopropanol was removed by pouring and replaced with 600 µl 70% ethanol. The gDNA pellet was washed by gentle pipetting. The gDNA was again pelleted by centrifuging at maximum speed for 2 min, after which ethanol was removed by pipetting. gDNA pellets were dried for 1 h and then rehydrated overnight in 100 µl to 300 µl of elution buffer (EB), depending on pellet size.

The extracted gDNA was assessed for quality. gDNA degradation was assessed by migrating approximately 75 ng of gDNA on a 0.7% agarose gel with SYBR Safe (Invitrogen) for 30 min at 100 V. Bands were checked for smearing, which would indicate degradation. The presence of salt and protein in the

samples was detected using a NanoDrop 2000 small-volume spectrophotometer (Thermo Fisher Scientific). Absorbance at 230 nm, 260 nm, and 280 nm was measured, and ratios were used to calculate acceptable levels of proteins (260 nm/280 nm) and salts (260 nm/230 nm). Guideline acceptable ratio for proteins ranges from 1.8-2.0, and for salts ranges from 2.0-2.2. However, these guidelines assume an average of the ratios for pure nucleotide solutions, and GC-rich *P. aeruginosa* gDNA is likely to have lower ratios due to the lower relative abundance of adenine, which has the highest absorbance at 260 nm. Therefore, we accepted protein ratios as low as 1.6 and salt ratios as low as 1.7.

gDNA concentration was determined using the QuantiFluor dsDNA System (Promega, UK), which binds fluorescent proteins to DNA to determine concentration relative to a standard curve of known Lambda phage DNA concentration. Stock solutions of the QuantiFluor dye were prepared according to the protocol instructions. gDNA is first diluted to 1:100 and 1:1000 in TE buffer, then 100 µl is mixed with then 100 µl QuantiFluor dye in a black 96 well microtitre plate. After 5 min of incubation, fluorescence intensity is read at 490 nm/510 nm emission/excitation. After determining gDNA concentration, the samples were diluted to approximately 50 ng/µl in 100 µl in EB, according to the specifications of the sequencing centre. The concentration was re-assessed using the QuantiFluor protocol. gDNA was stored at −20°C until delivery to the sequencing centre, and was delivered on dry ice to prevent degradation in transit.

8 Tables and Figures

Figure 1: A. Probability of fixation of invaders in RL and RH populations (data from Gifford and MacLean 2013). B. Distribution of fixation times of beneficial mutations in the invader lineages.

Figure 2: Mean competitive fitness of successful invaders (±SE). Invaders with beneficial mutations had higher fitness if they evolved in the presence of the high-evolvability resident (RH) over the low-evolvability resident (RL). Grey areas correspond to mean competitive fitness of the evolved resident ±SE.

Figure 3: A. Average maximum growth rate of the tester strain (±SE) correlates with manipulated environmental quality, when grown in the presence of the ancestral resident. B. Effective environmental degradation increased with resident fitness. C. Average fitness benefit (±SE) conferred by mutations was greater in a degraded environment for invaders with mutations from both the RL and RH populations.

Table 1: Mutations acquired by successful invaders. Gene ID, protein and genomic positions are relative to *Pseudomonas aerugionsa* reference genome (NC_002615.2).

Gene ID(s)	Function	Frequency	Mutations
Invaders in RL			
PA2449	transcriptional regulator	5	SNPs (D156G, P181L, R241H, L290Q, A452V)
PA2620, PA5042	ATP-binding protease component ClpA, type 4 fimbrial biogenesis protein PilO	1	substitution and 3 residue insertion (I362TRSV) frame shift (F49fs)
PA4941	protease subunit HflC	1	stop gained (Q28X)
intergenic		1	SNP (genomic position 3,470,748 A>G)
no mutations		7	–
Invaders in RH			
PA2449	transcriptional regulator	5	SNPs (R76L, N105S, A189V, A240V, V337L)
PA3711	probable transcriptional regulator	1	SNP (D6N)
PA4942	protease subunit HflK	1	frame shift (K54fs)
intergenic		1	deletion (genomic position 785,623—785,700)
no mutations		7	–